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13. ABSTRACT *(Maximum 200 words)*

Human alpha-fetoprotein (hAFP) upon incubation with sex steroid hormones is chemically converted into an inhibitor of estrogen-dependent breast cancer growth. This document describes the progress which was made during the first year of a research grant which was designed to develop the active form of hAFP as a treatment for breast cancer and establish markers which both predict and monitor breast cancer responsiveness to this form of AFP.

The full-length molecule of hAFP was produced from the recombinant human gene placed in an *E. coli* expression system. This product was found to be convertible to an active form which inhibited estrogen-dependent growth of human breast cancer grown as a monolayer in culture and as a xenograft in mice. The third domain of hAFP was produced from a recombinant, truncated gene placed in a baculovirus expression system. This product was activable and retained all of the activity found in the full-length molecule. A mammalian cell culture system was developed which produced hAFP already in its active form. This product was also quite effective in retarding breast cancer growth.

Further development of these products is underway, as is predicting and monitoring breast cancer responsiveness to the active form of AFP.

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James A Bennett 7-24-95

PI - Signature Date

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Introduction

(a) Nature of the problem

Epidemiological and literature studies strongly suggest that alpha-fetoprotein (AFP) is the endogenous factor in pregnancy which confers on parous women their significant reduction in risk of breast cancer. Our laboratory studies support this connection by demonstrating that natural human AFP undergoes a conformational change in the presence of ligands that have receptors in the steroid/thyroid hormone receptor superfamily; and this transformed product (*t*AFP) stops the growth of human breast cancer growing in culture and as xenografts. Our technical objective is to develop *t*AFP into a therapeutic agent for breast cancer. Recombinant (*r*) AFP has been produced for this purpose.

(b) Background

Epidemiology and literature studies showing the relationship between AFP and breast cancer. An experiment of nature suggests that alpha-fetoprotein (AFP) is the factor in pregnancy which confers on parous women their significant reduction in risk of breast cancer. As shown in Table 1a, AFP is elevated in maternal serum during pregnancy. Furthermore, there are factors in pregnancy such as maternal race, weight, hypertension, consumption of alcohol, number of fetuses *in utero*, and neural tube defect in the fetus where maternal serum AFP (MSAFP) is substantially altered from normal pregnancy levels. In studying the literature, we have found the consistent and striking correlation that in those pregnancy conditions associated with an elevated level of MSAFP, there was a significant reduction in the lifetime risk to the mother of acquiring breast cancer (1). Conversely, in pregnancy conditions characterized by low MSAFP (alcohol), subsequent breast cancer risk was elevated (Table 1a). We carried out epidemiologic studies analyzing retrospective data that extend and confirm the correlation between MSAFP levels and breast cancer risk (Table 1b). Recently Ekbom et al. (2) have published an epidemiological study which suggests that, at least in the case of hypertension during pregnancy, the reduction of breast cancer risk is also passed on to the fetus. He is in agreement with our speculation that it is AFP in the fetal and maternal circulation that protects the offspring as well as the mother against later development of breast cancer.

Table 1

Association of High Maternal Serum AFP with Decreased Breast Cancer Risk

Maternal Conditions 1		2	Maternal Serum AFP Concentration		Maternal Lifetime Breast Cancer Risk	
1a						
Pregnant	vs.	Non-pregnant	1 > 2	(3)*	1 < 2	(4)
Pregnant, black	vs.	Pregnant, white	1 > 2	(5)	1 < 2	(6)
Pregnant, lean	vs.	Pregnant, obese	1 > 2	(5)	1 < 2	(7)
Pregnant, consuming no alcohol	vs.	Pregnant, consuming alcohol	1 > 2	(8)	1 < 2	(9)
1b						
Pregnant, hypertensive	vs.	Pregnant, normotensive	1 > 2	(10)	1 < 2	(11)
Pregnant, with multiple fetuses	vs.	Pregnant, with a single fetus	1 > 2	(12)	1 < 2	(13)
Pregnant, fetus with neural tube defect	vs.	Pregnant, fetus no neural tube defect	1 > 2	(14)	1 < 2	(15)

*The numbers in the brackets are the reference sources for the data.

Growth regulation of hormone-dependent normal tissues by transformed AFP. We have carried out laboratory studies which support the above theory derived from epidemiological studies. AFP was isolated from human cord sera (16). Incubation of AFP with estrogen for one hour at room temperature generated a product which inhibited growth of estrogen-stimulated mouse uterus (Table 2). AFP alone did not inhibit growth.

Table 2

Inhibition of Estradiol (E_2)-Stimulated Mouse Uterine Growth[a] by Transformed Human AFP

Injectant 1	*1 hr* ⟶	Injectant 2	Mean Uterine[e] Weight ± S.E. (mg/g body wt)	Growth[f] Inhibition %
Saline		Saline	0.98 ± 0.04	
Saline		E_2[d]	1.61 ± 0.06	
E_2[d]		E_2	1.63 ± 0.06	
AFP/Sal[b]		E_2	1.60 ± 0.05	2
AFP/E_2[c]		E_2	1.43 ± 0.04[g]	31

[a] Immature (15-18 day old) female Nya:NYLAR mice were used for uterine growth experiments.
[b] 5 μg/ml AFP was added to an equal volume of saline and incubated at room temperature for 1 hr. 0.1 ml of this incubation mixture was injected i.p. into each mouse.
[c] 5 μg/ml AFP was added to an equal volume of 10 μg/ml E_2 and incubated at room temperature for 1 hr. 0.1 ml of this incubation mixture was injected i.p. into each mouse.
[d] 0.5 μg E_2 in 0.1 ml was injected i.p. to stimulate uterine growth.
[e] Twenty-two hours following administration of injectant 2, uteri were excised and uterine wet weights were determined. There were a minimum of 5 replicate mice per group.
[f] Percent growth inhibition was calculated as the AFP-induced reduction in E_2-stimulated uterine growth divided by the full E_2-stimulated increase in uterine growth multiplied by 100%.
[g] Significant inhibition, $p < 0.05$; Wilcoxon Sum of Ranks Test.

We have designated this product transformed AFP (*t*AFP), since all of the biological activity was contained in the large molecular weight (>10 kDa) fraction of the reaction mixture. A chemical reaction takes place during the incubation as evidenced by the time-dependent appearance of a difference spectrum in the ultraviolet region with λ max at 290 nm (17). The uniqueness of the reaction is demonstrated by the fact that incubation of AFP's closest homolog, fetal albumin, with estradiol under identical conditions did not result in a biologically active product (18) and did not produce a difference spectrum (17). Also, antibody to human AFP completely blocked generation of antiestrogenic activity (16). A molar excess of ligand to AFP was required to generate both the difference spectrum and the biological activity. As shown in Figure 1, ligands in addition to estrogens convert AFP to its growth-inhibitory form. These ligands have in common the fact that they all have receptors which belong to the steroid/thyroid hormone receptor superfamily (19). Hormones and vitamins that do not belong to this superfamily do not transform AFP. This suggests that the mechanism of action of *t*AFP is an interference with common biochemical pathways utilized by the receptors in this superfamily.

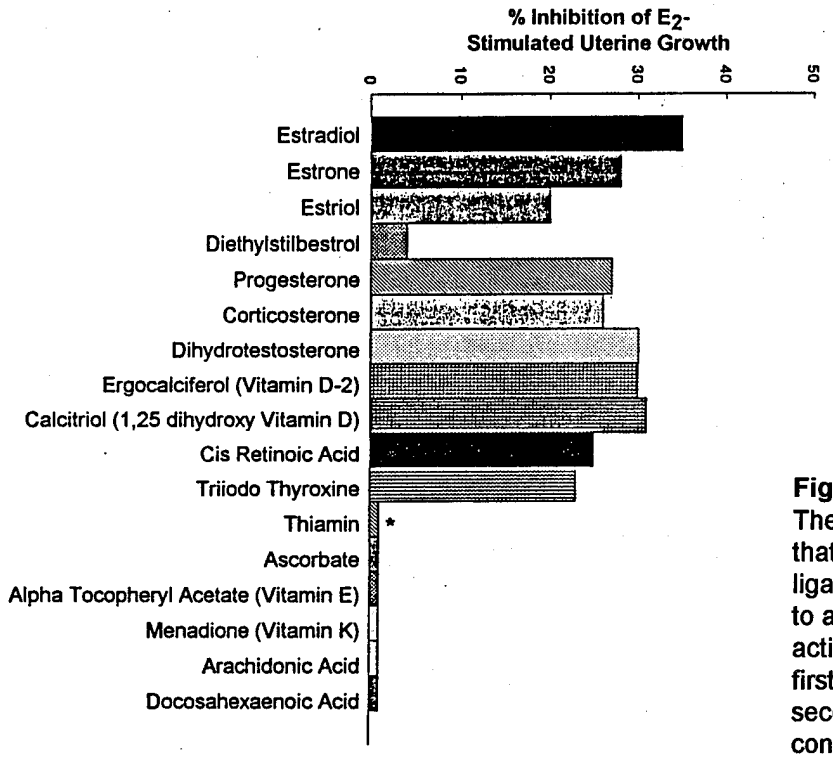


Fig. 1. Ligands used to activate AFP. The experimental design was similar to that described in Table 2, except that ligands other than estradiol were used to activate AFP. The control for ligand-activated AFP was ligand alone in the first injection followed by E_2 in the second injection. In no case were these controls inhibitory.

Growth inhibition of hormone-dependent breast cancer by tAFP. The estrogen-dependent MTW9A rat mammary tumor growing in syngeneic Wistar-Furth rats regressed during treatment with E_2-transformed AFP. Upon cessation of treatment, tumors resumed growth. Albumin treated with E_2 under similar reaction conditions did not inhibit tumor growth (Fig. 2) (20).

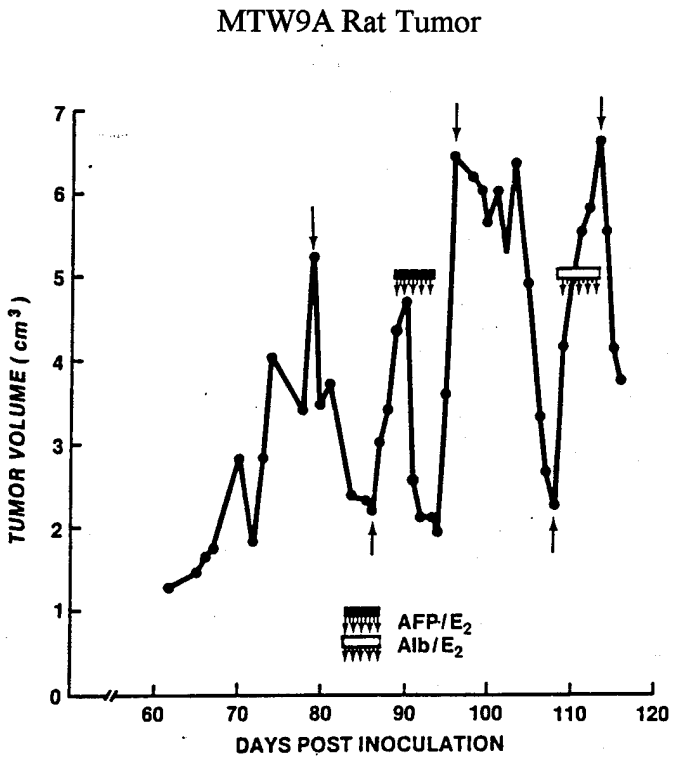


Fig. 2. Effect of AFP/E_2 on growth of the estrogen-dependent MTW9A rat mammary tumor in a syngeneic rat. Estrogen dependence of tumor growth was verified by removal (↓) and reinsertion (↑) of Silastic E_2 implants. Injections of AFP/E_2 (0.8 μg rat AFP/0.2 μg E_2, preincubated for 60 min) were given i.p. twice daily for 5 days in a volume of 0.1 ml. When similar quantities of albumin/E_2 were given to the same rat 20 days later, tumor growth was not affected.

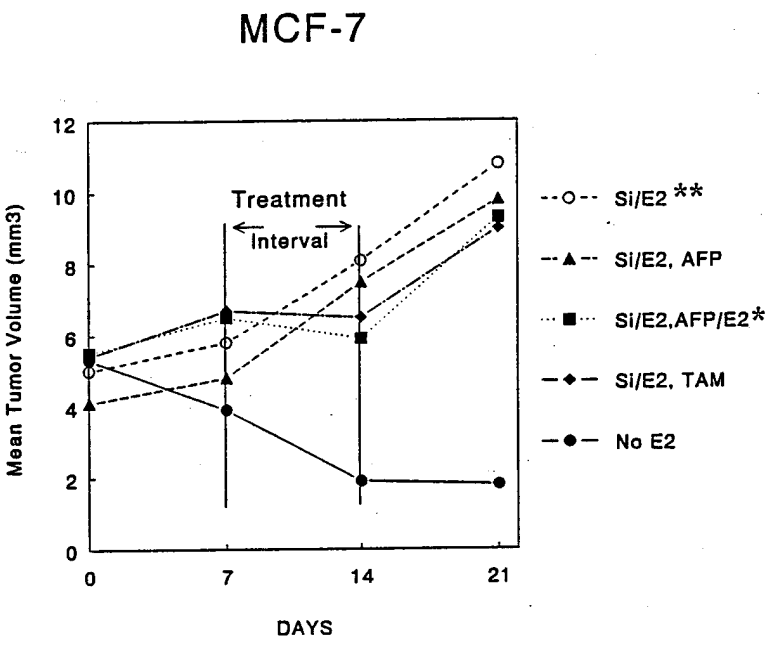


Fig. 3. Inhibition of MCF-7 breast cancer xenografts by *t*AFP.

* AFP/E_2 = *t*AFP

** Si/E_2 = 1 cm Silastic tubing filled with solid estradiol, sealed at both ends and implanted subcutaneously

Similarly, *t*AFP stopped the growth of estrogen-dependent human MCF-7 breast cancer (Fig. 3). These tumors were growing as xenografts under the kidney capsule of immunosuppressed, estrogenized mice. Daily treatment from day 7 to day 14 with E_2-transformed human AFP substantially inhibited tumor growth, resulting in no increase in tumor volume during treatment. In contrast, tumor volume increased by 55% during treatment with untransformed AFP. Tamoxifen, which is now standard treatment for estrogen-receptor-positive breast cancer, also substantially inhibited growth of these tumor xenografts. Its potency was similar to that of *t*AFP.

Recombinant AFP has biological activity similar to that of natural AFP. It became clear to us that the supply of natural AFP would not be sufficient for clinical trials. Therefore, we looked for a source of recombinant AFP that could be tested for biological activity in our bioassays. Dr. K. Muira (KRI International, Japan) supplied us with Domain I of human AFP expressed in *E. coli*, and Dr. Shinzo Nishi (Hokkaido University, Sapporo, Japan) supplied us with full-length human AFP expressed in yeast. As shown in Table 4, Domain I of human AFP was not activable to antiestrogenic activity. However, full-length human AFP expressed in yeast had substantial antiuterotrophic activity. Unfortunately, Dr. Nishi encountered production problems with the recombinant human AFP expressed in yeast and was not able to supply us with additional AFP. Recently we established a collaboration with Dr. Robert Murgita (McGill University, Montreal, Canada). He has been able to supply us with recombinant human AFP-full length molecule, expressed in an *E. coli* system and in the baculovirus system. In the baculovirus system, AFP is glycosylated, whereas in the *E. coli* system, it is not. As shown in Table 3, all of the full-length molecules could be activated to antiestrogenic activity.

Table 3

Inhibition of E_2-Stimulated Mouse Uterine Growth by Different Sources of Transformed Recombinant AFP

AFP Source	*% Inhibition of E_2-Stimulated Mouse Uterine Growth*[4]
Human Cord Sera	31
Yeast; Recombinant Human, Full-length[1]	45
E. coli; Recombinant Human, Full-length[2]	28
E. coli; Recombinant Human, Domain I[3]	0
Baculovirus/Sf9 cells; Recombinant Human, Full-length[2]	22
Mouse Amniotic Fluid	42
E. coli; Recombinant Mouse, Full-length[2]	31
Baculovirus/Sf9 cells; Recombinant Mouse[2]	26

[1]Obtained from Dr. Shinzo Nishi, Hokkaido University, Sapporo, Japan
[2]Obtained from Dr. Robert Murgita, McGill University, Montreal, Quebec, Canada
[3]Obtained from Dr. K. Miura, KRI International, Tokyo, Japan
[4]AFPs at doses of 1 μg, 0.5 μg or 0.25 μg, either transformed by 0.5 μg E_2 or in its native state, were tested in the mouse uterus bioassay. 0.25 μg was an optimal inhibitory dose for *t h*AFP, 1.0 μg was optimal for *t m*AFP. Untransformed or native AFP resulted in values of less than 8% inhibition.

Support from this grant will enable us to continue evaluation of recombinant human AFP as an oncostatic agent against breast cancer cells growing in culture and as xenografts *in vivo*. Dr. Murgita has agreed to supply us with an amount of recombinant human AFP that will be more than sufficient to complete the aims of this study. Moreover, he is highly committed to the development of AFP all the way to clinical trials for both immunoregulatory diseases, which is his interest, and for breast cancer and perhaps other endocrine cancers, which is our interest. However, to be absolutely sure that the supply of recombinant AFP does not become limiting, as was the case in our collaboration with Dr. Nishi, my collaborators at Albany Medical College have begun to produce recombinant human AFP in the baculovirus expression system. The fact that recombinant AFP possesses this antioncotic property indicates that we have a means through which AFP can be produced in sufficient quantity for clinical trial.

Physiological relevance of tAFP. Although the research proposed in this document is focused on the pharmacological application of *t*AFP for the treatment of breast cancer, the epidemiological and laboratory studies described above suggest a physiological relevance for *t*AFP. AFP is secreted primarily by the fetal liver, is a major protein component of fetal plasma and crosses the placenta into the maternal circulation (21). In pregnant women, estrogen, progestins and androgens are produced primarily by the placenta and readily cross into the fetal circulation (22). Thus the conditions exist *in utero* for reaction of AFP with sex steroid hormones to produce *t*AFP in the fetal plasma. The physiological role of AFP, and especially *t*AFP, may be to act as a rudimentary servo mechanism which desensitizes endocrine tissues to inappropriately high levels of hormones which occur during gestation, since the fetus develops in the presence of a large concentration of maternal and placental hormones, has receptors for these hormones, but early on does not have the sophisticated control mechanisms of late fetal or adult life to regulate

the production of and response to these hormones. A "side effect" would occur when *t*AFP crossed the placenta into the maternal circulation where it would extinguish microscopic premalignant and/or cancerous foci in the breast that later on in life would be promoted to clinically detectable breast cancers. Such a "side effect" would explain the epidemiological data described in Table 1.

(c) Hypothesis/Purpose

Our working hypothesis is that AFP interacts with ligands in the steroid/thyroid hormone receptor superfamily and undergoes a conformational change, producing a growth-regulatory molecule which stops the growth of hormone-receptor-positive breast cancer. The studies proposed in this application have as their purpose the generation of requisite preclinical data which will underpin the clinical application of ligand-transformed AFP (*t*AFP) in patients with breast cancer. The specific aims are:

(d) Specific Aims/Technical Objectives

1. Transform recombinant AFP to its active state in a way that both maximizes its antitumor activity and sustains its lack of host toxicity. This will entail selecting the appropriate activating ligand. For activation it seems wise to avoid ligands that themselves could be trophic for the breast, such as estrogens and progestins. Therefore the focus will be on vitamin A analogs and vitamin D analogs, since (a) the parent compounds in these families are as effective as estradiol in converting AFP to its active form (Fig. 1); (b) both of these ligand families when used alone decrease proliferation and increase differentiation of certain cancers, including breast cancers, and are themselves beneficial; and (c) there is extensive clinical experience with these agents.
2. Identify cellular markers that predict tumor responsiveness to *t*AFP. This will entail evaluating characteristics in the tumor, such as tissue type, level of sex steroid hormone receptors, level of AFP receptor and level of IGF receptor, and correlating these tumor markers with the growth-inhibitory response to *t*AFP.
3. Develop non-invasive tests that monitor biological activity of *t*AFP in the host. A decrease in serum levels of IGF_1, an increase in circulating FSH and LH, and a decrease in serum levels of breast-cancer-associated antigen (CA 15-3), would all be indicative of *in vivo* biological activity of *t*AFP and are rational choices to use as intermediate markers of tumor response to *t*AFP.
4. Characterize the cell cycle changes and type of growth arrest induced by *t*AFP. In this aim we will determine whether *t*AFP causes cells to accumulate in a particular phase of the cell cycle, whether cell death accompanies growth arrest during long-term treatment with *t*AFP, whether cell death is via apoptosis, necrosis or a combination of both, and whether the information garnered from these experiments can be used to synergistically combine *t*AFP with other agents.

Other aims that are important but are not a part of this proposal are study of the mechanism of action of *t*AFP at the molecular level in the tumor target cell, identification of the active site on the *t*AFP molecule, and genetic engineering and/or peptide synthesis of a smaller molecule that bears the active site and has antitumor activity. These studies are being carried out by my colleagues at Albany Medical College and at the Wadsworth Center for Laboratories and

Research of the New York State Department of Health, and support for these studies is being requested in separate grant proposals. We have assembled a team of intramural and extramural scientists with a broad spectrum of essential skills to develop, understand and apply *t*AFP as a new agent for the prevention and treatment of breast cancer. To the best of our knowledge, we are the only group in the U.S. pursuing this unique property of AFP.

Body

a. Methods and Results Obtained

Three sources of AFP were evaluated for their ability to be transformed into inhibitors of estrogen-stimulated human breast cancer growth. The three sources were 1] recombinant human AFP produced in an *E. coli* expression system (ErhAFP) by our collaborators at McGill University, 2] recombinant human AFP (Domain III) produced in a baculovirus expression system (BrhAFP) by coinvestigators on the grant who are at Albany Medical College, and 3] natural human AFP (NhAFP) secreted from a human hepatoma carried in culture at Albany Medical College.

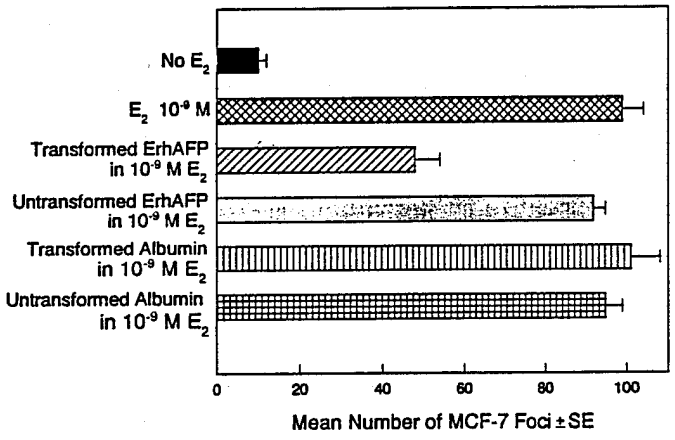
Results with ErhAFP. Several batches of ErhAFP were prepared and tested for antiestrogenic activity. Our primary screen was inhibition of estrogen-stumulated mouse uterine growth described earlier in Table 2, because of its quick turn-around time, low cost and in vivo nature. As shown in Table 4, only batch 18 provided reliable antiestrogenic activity.

Table 4
Effect of Different Batches of E_2-Transformed ErhAFP on Estrogen-Stimulated Mouse Uterine Growth

Batch of ErhAFP	% Inhibition of E_2-Stimulated Mouse Uterine Growth (replicate experiments)[a]
14	7
15	13
16	28,* 20
17	0
18	38,* 33,* 33,* 39* [b]
19	39,* 28

[a] Inhibition of E_2-stimulated mouse uterine growth was established with all the controls described in Table 2 for each experiment.
[b] Significant inhibition, $p < 0.05$, Wilcoxon Sum of Ranks Test.

The other batches were just as pure as 18 and by all criteria (molecular weight, reactivity with anti-AFP and sequencing of N-terminal amino acids) identified as AFP. The basis for their lack of activity is not clear. One possibility is that in isolating the AFP from *E. coli*, the material may have been exposed for too long a time to harsh chemical conditions required to lyse the *E. coli* and break down the inclusion bodies where the AFP was stored. Of all the batches, 18 was exposed to these conditions for the least amount of time.



Batch 18 was evaluated for its ability to inhibit estrogen-stimulated growth of human MCF-7 breast cancer cells into postconfluent foci. As shown in Figure 4, transformed ErhAFP significantly inhibited the growth of human MCF-7 breast cancer cells in culture, whereas untransformed AFP, albumin and albumin exposed to transforming conditions were without effect.

Fig. 4. Inhibition of growth of MCF-7 Breast cancer cells in culture by *t*ErhAFP.

Erh AFP was then tested against MCF-7 human breast cancer growing as a xenograft under the kidney capsule of immune-deficient mice. Tumors were implanted and growth was established for 10 days prior to treatment.

As shown in Figure 5, the tumor was completely dependent on estrogen (Silastic subcutaneous implants containing estradiol, Si/E_2) for growth. Treatment was carried out from day 10 to day 20 after implantation by daily i.p. administration of test agents. E_2-transformed ErhAFP significantly inhibited tumor growth during the treatment interval. However, tumor growth resumed upon cessation of treatment. This was similar to the effect of tamoxifen, which was evaluated as a positive control in the same experiment. Untransformed AFP was without effect.

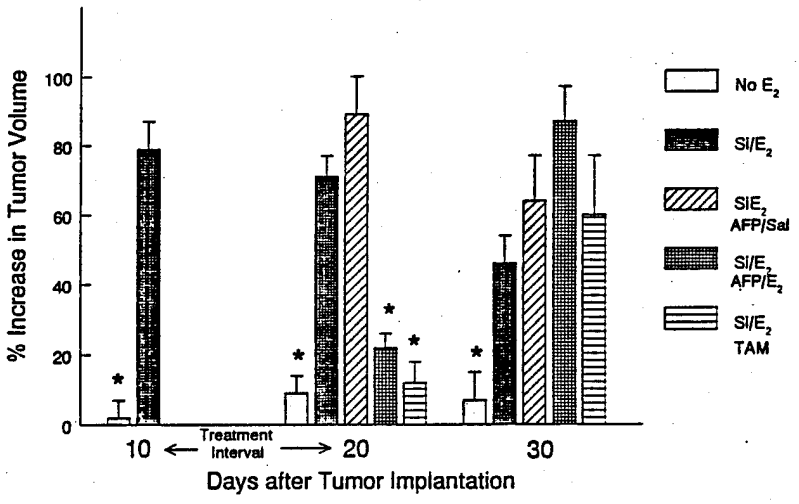


Fig. 5. MCF-7 with *t*ErhAFP (treatment interval days 10-20)

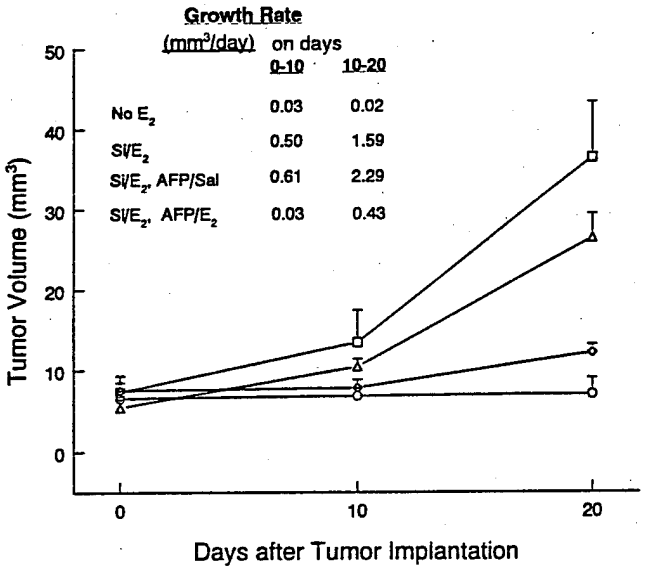


Fig. 6. MCF-7 with *t*ErhAFP (treatment interval days 0-20)

The impact of earlier and more prolonged treatment was assessed, and the results are shown in Figure 6. If treatment was initiated at the time of tumor implantation and continued daily for 20 days, significant growth inhibition was obtained. Inhibition was greater for the first 10 days of treatment compared to the second 10 days of treatment. Upon examination of tumors during survival laparotomy required for measurements of tumor size, there was no indication that neovascularization had been inhibited. In fact, gross as well as histological assessments were consistent with viable tumor in a state of cytostasis as a result of treatment.

In contrast, the estrogen-receptor-negative MDA-MB-231 human breast cancer was not dependent on estrogen for growth and was not inhibited by either transformed ErhAFP, untransformed ErhAFP, or tamoxifen (Figure 7). This suggests that the mechanism of action of transformed AFP is through the estrogen receptor. However, additional ER-positive and ER-negative human breast cancers need to be evaluated to establish this point.

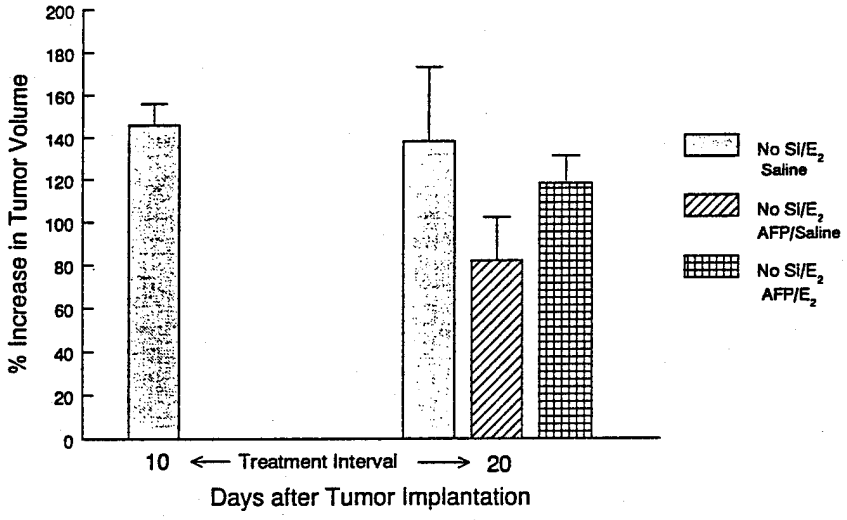


Fig. 7. MDA-MB-231 with *t*ErhAFP (treatment interval days 10-20)

In the above experiments batch 18 Erh AFP was transformed by incubation with E_2 to an inhibitor of estrogen-stimulated growth. We had preliminary data that retinoids could also transform hAFP to an inhibitor of estrogen-dependent mouse uterine growth (23). Since retinoid-transformed AFP would be more appropriate than E_2-transformed AFP for clinical use against breast cancer, experiments were carried out to evaluate the ability of retinoids to transform ErhAFP to an inhibitor of estrogen-dependent growth. Several concentrations of batch 18 ErhAFPwere incubated with 5 μg/ml 13-cis-retinoic acid (13cRA) for one hour at room

temperature. As shown in Table 5, the reactant ratio of 2.5 μg/ml AFP to 5 μg/ml 13cRA yielded a product with antiestrogenic activity similar to that obtained when E_2 was used as the activating ligand. AFP alone and 13cRA alone had no effect on E_2-stimulated growth of mouse uterus.

Table 5
Inhibition of E_2-Stimulated Mouse Uterine Growth by Retinoid-Transformed ErhAFP

Injectant 1	*1 hr* ⟶	Injectant 2	Mean Uterine Weight ± SE (mg/g body wt)	Growth Inhibition %
Saline		Saline	0.98 ± 0.06	
Saline		E_2 [f]	1.71 ± 0.07	
Saline		13cRA [a]	0.99 ± 0.03	
13cRA [a]		E_2 [f]	1.70 ± 0.05	
AFP/Sal [b]		E_2	1.73 ± 0.07	
AFP/13cRA [c]		E_2	1.61 ± 0.07	14
AFP13cRA [d]		E_2	1.49 ± 0.10 [g]	30
AFP/13cRA [e]		E_2	1.52 ± 0.05	26

[a] 0.5 μg 13-cis-retinoic acid (13cRA) in 0.1 ml was injected i.p.
[b] 5 μg/ml AFP was added to an equal volume of saline and incubated at room temperature for 1 h. 0.1 ml of this mixture was injected i.p. into each mouse.
[c] To 20 μg/ml AFP in 1 ml was added 25 μl of a 200 μg/ml solution of 13cRA, and these were incubated together for 1 h at room temperature. 0.1 ml of this mixture was injected into each mouse.
[d] As in c, except AFP was at a concentration of 2.5 μg/ml.
[e] As in c, except AFP was at a concentration of 0.25 μg/ml.
[f] 0.5 μg E_2 in 0.1 ml was injected i.p. to stimulate uterine growth.
[g] Significant inhibition, $p < 0.05$; Wilcoxon Sum of Ranks Test.

Our colleagues at McGill University have run out of batch 18 ErhAFP. They have licensed the rights to their patent on their method of production and purification of ErhAFP to Atlantic Biopharmaceuticals, Inc. This company has invested in the materials needed to make large quantities of ErhAFP, and they are presently scaling up production of this protein in anticipation of clinical trials for use in certain autoimmune diseases (rheumatoid arthritis and myasthenia gravis), which is their interest, and in breast cancer if our results warrant an application in breast cancer. As this new material becomes available, we will continue testing its biological activity in our breast cancer assays according to the aims in this grant application. In the meantime, we have begun testing two sources of AFP produced at Albany Medical College. One is domain III of human AFP produced in a baculovirus expression system (BrhAFP), and the other is natural human AFP secreted by a human hepatoma grown in tissue culture (NhAFP).

BrhAFP. The gene for full-length human AFP was not commercially available. However, the cDNA for Domain III of human AFP was available and was obtained from the American Type Culture Collection. We decided to test Domain III of AFP for activity before trying to isolate the gene for the full-length molecule. As described below, we were quite fortunate in that Domain III contained all of the activity found in the full-length molecule. Prior to describing those results, let me take you through our method for production and purification of Domain III of human AFP.

The desired cDNA was amplified using PCR and subcloned into TA cloning vector (Invitrogen) in order to provide convenient "sticky" ends. This subcloning step permitted directional cloning of our cDNA into a baculovirus transfer vector downstream of a polyhedron promoter and flanked by viral sequences necessary for allelic replacement. Transfer vectors were modified to include a segment which codes for a polyHistidine region and a thrombin cleavage site on the N-terminus of each polypeptide.

Sf9 cells growin in 25 cm^2 flasks were cotransfected with 1 μg of BaculoGold linear viral DNA (Invitrogen, Inc.) containing a lethal mutation and with 2 μg of recombinant baculovirus transfer vector and incubated for 4 h in order to allow allelic replacement of the lethal mutation. After growing in complete medium for 4-6 days, media containing virus was harvested and stored at 4°C. Virus production was monitored by Western blot analysis (for AFP) and titered by plaque assay. Virus was amplifed about 400 fold in each of two subsequent passages through Sf9 cells, in order to provide enough virus for large-scale production of protein. Sf9 cells were also used for the large-scale protein production. These cells were removed from the culture medium by centrifugation, and the non-secreted fusion protein (His-tagged AFP) was then solubilized and purified by passing the crude preparation over an affinity column to which the polyHis linker binds, allowing undesired proteins to pass through unretarded by the column. His-tagged AFP was then eluted from the column with imidazole, the linker was removed (by addition of thrombin, followed by dialysis), and AFP identity was confirmed by SDS-PAGE, Western blot, amino terminal protein sequencing, and amino acid analysis.

This material was tested for activability to antiestrogenic activity in the immature mouse uterus bioassay. As shown in Table 6, E_2-transformed BrhAFP significantly inhibited E_2-stimulated growth of immature mouse uterus. Untransformed BrhAFP was not inhibitory.

Table 6

Inhibition of E_2-Stimulated Mouse Uterine Growth by E_2-Transformed BrhAFP

Injectant 1	*1 hr* ⟶	Injectant 2	Mean Uterine Weight ± SE (mg/g body wt)	Growth Inhibition %
Saline		Saline	0.82 ± 0.08	
Saline		E_2	1.53 ± 0.05	
E_2		E_2	1.57 ± 0.08	
AFP/Sal [a]		E_2	1.64 ± 0.11	
AFP/E_2 [b]		E_2	1.36 ± 0.04 [c]	28

[a] 5 μg/ml of BrhAFP was mixed with an equal volume of saline for 1 h at room temperature, and 0.1 ml of this mixture was injected into the mice.

[b] 5 μg/ml of BrhAFP was mixed with an equal volume of 10 μg/ml E_2 for 1 h at room temperature, and 0.1 ml of this mixture was injected into the mice.

[c] Significant inhibition, $p < 0.05$, Wilcoxon Sum of Ranks Test.

Similarly, E_2-transformed BrhAFP significantly inhibited E_2-stimulated growth of human McF-7 breast cancer cells into postconfluent foci, and as expected, untransformed BrhAFP was not inhibitory (Figure 8).

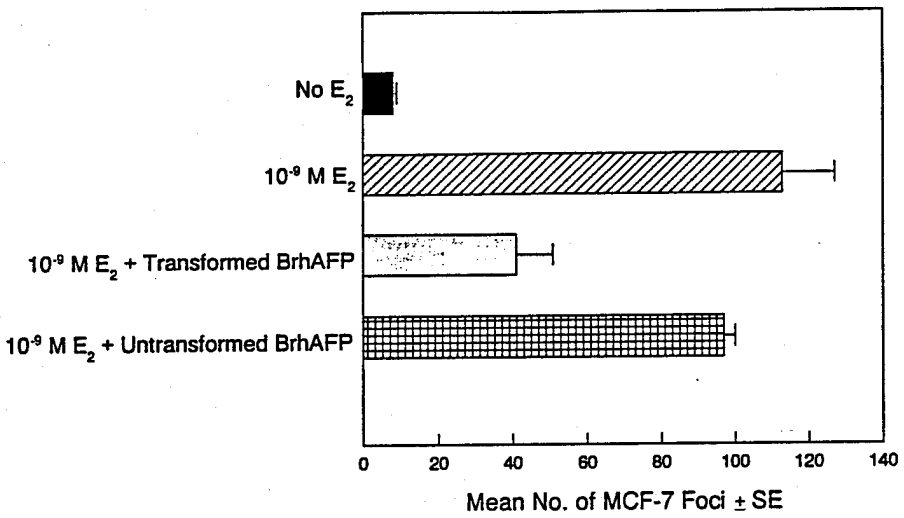


Fig. 8. Inhibition of growth of MCF-7 breast cancer cells in culture by *t*BrhAFP

NhAFP. Experiments were carried out to test our theory that E_2-mediated activation of AFP can take place under physiological conditions in utero. In order to mimic those conditions, a human hepatoma (Hep G-2) which actively secretes AFP was grown in serum-free medium in the presence or absence of E_2 at a concentraiton of 10^{-9} M. After 4 days of culture, supernatant was harvested and tested in the immature mouse uterus bioassay and the MCF-7 foci assay. As shown in Table 7, crude supernatant from hepatoma cells grown in 10^{-9} M E_2 significantly inhibited E_2-stimulated growth of mouse uterus, while supernatant from cells grown in the absence of E_2 was not inhibitory. The concentrations of AFP in these two supernatants were similar, suggesting that the presence of physiological levels of E_2 in the Hep G-2 growth medium uniquely resulted in the

formation of a growth-regulatory product. We believe this product is the antiestrogenic form of AFP, and we are carrying out the requisite experiments to substantiate this hypothesis.

Table 7
Inhibition of E_2-Stimulated Mouse Uterine Growth by the Culture Supernatant from Hep G-2 Human Hepatoma

Injectant 1	*1 hr* ⟶	Injectant 2	Mean Uterine Weight ± SE (mg/g body wt)	Growth Inhibition %
Saline		Saline	0.85 ± 0.04	
Growth Medium		Saline	0.91 ± 0.06	
Growth Medium with 10^{-9} M E_2		Saline	0.98 ± 0.06	
Growth Medium		E_2	1.53 ± 0.08	
Growth Medium with 10^{-9} M E_2		E_2	1.58 ± 0.06	
Culture Supernatant [a]		E_2	1.66 ± 0.08	
Culture Supernatant [b] with 10^{-9} M E_2		E_2	1.35 ± 0.08 [c]	38

[a] This culture supernatant contained 68 μg/ml. 0.2 ml of this crude supernatant was injected i.p. into each mouse.
[b] This culture supernatant contained 56 μg/ml. 0.2 ml of this crude supernatant was injected i.p. into each mouse.
[c] Significant inhibition, $p < 0.05$, Wilcoxon Sum of Ranks Test.

No additional incubation with high concentrations of E_2 was required to achieve this inhibitory activity. This is important because the concentrations of E_2 in the supernatant are physiological and would be well tolerated in vivo. This may obviate our need to explore other activating ligands, a key component in the first aim of this proposal. These supernatants were also tested against MCF-7 cells growing in culture. Again, the supernatant from hepatoma cells growing in 10^{-9} M E_2 was inhibitory and the other supernatant without E_2 was not (Figure 9).

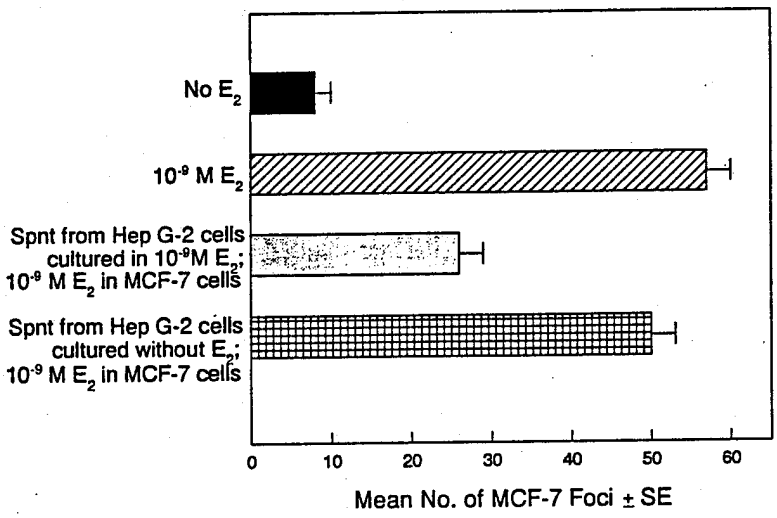


Fig. 9. Inhibition of MCF-7 breast cancer cells in culture by NhAFP.

We have not yet proven that the activity in this supernatant is due to AFP, but these experiments are currently under way.

Work has begun determining receptor content of tumor lines that will be used in this study. Receptors for estrogen, progesterone and androgen have been determined. Receptors for AFP and IGF-1 will be determined during year 2. Assays were performed by radioligand binding assay as described in the manuals for RIANEN Assay Systems (NEN DuPont). Cytosols were prepared by homogenization of cell pellets of cell lines obtained from culture, followed by ultracentrifugation at 65,000xg for 1 hour. After analysis of cytosols for protein content (Biorad Systems), portions were combined with the appropriate tritium-labeled steroid hormone (with and without excess radioinert steroid inhibitor) and were incubated overnight at 4°C. After adsorption of unbound steroid with dextran-coated charcoal and clarification by low-speed centrifugation, their content of protein-bound tritium was determined by liquid scintillation counting. Receptor content for each tumor line was calculated from Scatchard plots of the data, and the results are shown below in Table 8.

Table 8

Estrogen, Progestin and Androgen Receptors in Tumor Cell Lines

	fmol/mg cytosol protein		
	ER	PR	AR
MCF-7 (breast)	117	96	281
T47D (breast)	621	381	356
MDA (breast)	0	57	N.D.
HS578T (breast)	0	31	250
LNCaP (prostate)	309	106	600

N.D. Not determined due to insufficient quantity of cytosol protein

Conclusions

a. ***Implications of Work.*** We have established that recombinant AFP can be transformed by incubation with sex steroid hormones or retinoids into an inhibitor of E_2-stimulated human breast cancer growth. This important milestone not only shows that recombinant material has anticancer activity similar to that of the natural material, but also gives credence that the requisite quantity of material for clinical trial can be achieved. In addition, it was found that the active site in AFP responsible for its antiestrogenic function is contained within the third domain of the AFP molecule. The fact that full activity is contained in the third domain of the molecule suggests that even smaller portions of the molecule may retain activity and lead to a more stable and perhaps synthetic product. Conversion to its antiestrogenic form is a rather labile property of both the recombinant and natural isolates of AFP. It is destroyed by harsh conditions of pH, temperature, exposure to chaotropic agents and exposure to denaturants. Therefore, conditions of production, purification and storage must be carefully controlled to preserve this property of the molecule.

We have also established that the active form of AFP can be produced in mammalian cell culture when a human liver cancer which secretes AFP is grown in the presence of physiological levels of E_2. This adds support to our hypothesis that the active form of AFP is produced in utero where the concentrations of AFP and E_2 are very similar to those found in our culture medium. We speculate that the physiological basis for this product is to dampen the response of fetal tissues to sex steroid hormones which are prevalent during gestation. The epidemiological data showing a relationship between increased AFP and reduced breast cancer incidence can be explained by E_2-transformed AFP crossing the placenta, entering the maternal circulation and extinguishing premalignant foci in the breast that if left unabated would be promoted toward malignancy under the influence of estrogen.

b. ***Future Work.*** Our future work will continue to be directed toward isolating and capturing the active form of AFP so that it can be studied as a treatment for breast cancer. One of the first priorities in year 2 will be to chronically treat mice bearing the estrogen-dependent human MCF-7 breast cancer xenograft with one of our active forms of AFP and assess through histomorphometric studies the type of damage (lethal or non-lethal) done to the tumor. Also, the assays for AFP receptor and IGF_1 receptor will be developed, and these receptors will be quantitated in the tumors described in Table 8 on Page 19 of this report. We will then begin treating a variety of these tumors with the active form of AFP to determine their sensitivity to this agent and to assess whether their receptor status predicts their sensitivity to AFP. By the end of year 2 we will have a large supply of reliably active AFP that will be used to complete the remaining aims of our original grant proposal.

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